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Receptor homodimerization plays a critical role in a novel dominant negative P2RY12 variant identified in a family with severe bleeding

Short title: New dominant negative P2RY12 variant

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Essentials.
- 3 groups recently identified dominant *P2RY12* variants
- Absence of phenotype/genotype correlation for the previously reported dominant P2RY12 variants
- Proline plays an important role in P2Y12R ligand binding and signaling defects
- P2Y12R homodimer formation is critical for the receptor function and signaling

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Summary

Background: Although inherited platelet disorders are still underdiagnosed worldwide, advances in molecular techniques are improving disease diagnosis and patient management.

Objective: To investigate a Caucasian family with easy bruising and serious bleeding requiring transfusion following procedures.

Methods: Full blood count, platelet aggregometry, flow cytometry and western-blotting were performed before next-generation sequencing (NGS). Detailed molecular analysis of the identified P2Y12R receptor's variant was subsequently performed in mammalian cells over-expressing receptor constructs.

Results: All three referred individuals had markedly impaired ADP-induced platelet aggregation with primary wave only despite normal total and surface P2Y12R expression. By NGS, a single P2RY12:c.G794C substitution (p.R265P) was identified in all affected individuals and confirmed by Sanger sequencing. Mammalian cell experiments with the R265P-P2Y12R variant showed normal receptor surface expression versus wild-type (WT). Agonist-stimulated R265P-P2Y12R function (both signaling and surface receptor loss) was significantly reduced versus WT. Critically, the R265P-P2Y12R variant acted in dominant negative manner with agonist-stimulated WT-P2Y12R activity significantly reduced by variant co-expression suggesting dramatic loss of WT homodimers. Importantly, platelet P2RY12 cDNA cloning and sequencing in 2 affected individuals also revealed a 3-fold mutant mRNA overexpression, decreasing even further the likelihood of WT homodimer formation. R265 located within extracellular loop 3 (EL3) is one of four amino acids important for the receptor functional integrity, maintaining the binding pocket conformation and allowing rotation following ligand binding.

Conclusion: This novel dominant negative variant confirms the important role of R265 in EL3 in the functional integrity of the P2Y12R and suggests that pathological heterodimer formation may underlie this family bleeding phenotype.

Introduction

The P2Y12 receptor (P2Y12R), a major G-protein-coupled receptor (GPCR) in platelets, is one of the two platelet receptors for ADP stabilizing platelet aggregation and potentiating platelet activation initiated by many platelet agonists. Since its first observation[1], P2Y12R deficiency (MIM #609821) has been identified as an autosomal recessive disorder characterized by mild to moderate mucocutaneous bleeding and excessive bleeding in response to trauma or after surgery[2].

Over the years, different gene alterations affecting P2Y12R function leading to platelet dysfunction have been reported although such variations are relatively rare. P2Y12R defects can be associated with loss of expression (quantitative disorder) or loss of function (qualitative disorders). Rare naturally occurring loss of function variants in the P2RY12 gene offer a powerful approach to determine the P2Y12R structure–function relationships with receptor variations able to be studied in native cells, such as platelets, from affected individuals. Clinical evaluation of affected individuals also provides insights into the biological consequences of the P2Y12R dysfunction. To date a series of function-disrupting mutations have been identified and shown to affect multiple aspects of platelet P2Y12R function including surface receptor expression, ligand binding, G protein-coupling, receptor internalization and receptor sorting (reviewed in[3] and[4]). In 2007, Remijn and colleagues reported the first *P2RY12* dominant mutation within the receptor EL2 domain in a patient with abnormal ligand binding[5]. Soon after, another dominant mutation was reported at the junction between TM6 and EL3 in a patient with normal ligand binding but abnormal function (impaired signaling)[6] and more recently a dominant mutation affecting the PDZ binding domain, critical for the receptor recycling[7]. However, the mechanism correlating the phenotype to the genotype is still unknown.

The P2Y12R, like all Class A GPCRs, shares a similar structure comprising one extracellular N-terminal domain, one intracellular C-terminal domain and multiple transmembrane domains (TM) and extracellular loops (EL) [8]. The ability of GPCRs to form dimers/oligomers is widely accepted although their functional significance, especially *in vivo* still remains controversial [9-12]. We have recently demonstrated that two loss-of-function variants of another platelet expressed GPCR, the thromboxane TPα, display aberrant homo-dimer formation and this might represent one molecular mechanism through which platelet TPα receptor dysfunction affects the patient(s) carrying these mutations[13].

In this study of a Caucasian family with an inherited bleeding disorder, we identified a *P2RY12* mutation, predicting an arginine to proline (R265P) substitution within the receptor EL3 domain. We assessed the impact of this variant on P2Y12R function in both platelets from affected individuals and in cell lines and highlight a potential important role of P2Y12R homodimerization in receptor signaling.

Methods

Patients

The Caucasian family described participated in a human research ethic committee approved study using a gene panel to identify mutations associated with inherited platelet disorders. All individuals provided written informed consent and research was conducted in accordance with the Declaration of Helsinki.

Genetic studies

DNA was extracted from buffy coats by the salting-out method. Genotyping was performed using the Illumina Miseq platform as per manufacturer's instructions and a designed panel of 32 platelet genes (Illumina, Scoresby, VIC, Australia). Pathogenic variants or variants of uncertain significance were

confirmed by Sanger sequencing. P2RY12 exon 2 sequencing was performed using the forward primer 5'-GGGCTAAGATTCTCTCTGTTGTC and reverse primer 5'- GCGCTTTGCTTTAACGAGTTCTG with BioTaq DNA polymerase (Bioline, Lane Cove NSW, Australia). The sequence of the amplified fragment was obtained by capillary electrophoretic sequencing (AGRF, Sydney, NSW, Australia)

Platelet light transmission aggregometry (LTA)

Platelet-rich plasma (PRP) was prepared and agonist-induced LTA was performed on a four channel aggregometer (Chrono-Log Corporation, Haverton, PA, USA) as previously described[14] and following the ISTH-SSC recommendations for the standardization of light transmission aggregometry[15].

Platelet surface receptor expression and platelet activation measurement by flow cytometry

Flow cytometry was performed on a four-color FACScalibur flow cytometer (BD Biosciences, North Ryde, NSW, Australia) using antibodies from BD Biosciences (North Ryde, NSW, Australia) or Beckman Coulter (Lane Cove, NSW, Australia). Platelet surface glycoprotein expression levels were measured using EDTA PRP with a two-step antibody labeling technique: either mouse immunoglobulins or specific mouse monoclonal antibodies were incubated with PRP samples for 15 min at room temperature (RT), before the addition of Alexa Fluor 488-conjugated secondary goat-anti-mouse IgG antibody (Thermofisher, Scoresby, Vic, Australia) for a further 15 min-incubation at RT. Platelet surface expression of P-selectin (CD62P), a marker of alpha-granule release, Pac-1 antibody binding (fibrinogen mimetic) and platelet-monocyte aggregates were measured as previously described, with or without ADP (Morel-Kopp, JTH 2009). Data from 10,000 events were collected in a logarithmic mode for platelets and linear mode for leukocytes and analyzed using CellQuest software (BD Biosciences). In experiments assessing P2Y12R surface expression washed platelets were prepared and fixed, then labelled with primary anti-P2Y12 (1:75) or anti-DOK6 (negative control) antibodies followed by the fluorescent secondary Alexa Fluor 647

antibody (1:200). Surface P2Y12R levels were quantified in terms of geometric mean fluorescence intensity (MFI) from a gated population of 10,000 platelets after accounting for non-specific binding in negative controls. Data displayed show mean of three replicates.

Western-blotting

Platelet lysates were prepared and platelet western-blotting experiments were conducted as previously described[16]. Briefly, platelet lysate proteins were separated by SDS-PAGE, and subsequently transferred onto a nitrocellulose membrane. After blocking in 5% bovine serum albumin membranes were labelled overnight with the following primary antibodies rabbit polyclonal anti-P2Y12 (Novus Biologicals, Abingdon, UK) and mouse monoclonal anti-α-tubulin (Sigma, Gillingham, UK), diluted by a factor of 500 and 10,000 respectively. Bands were detected using appropriate secondary HRP-conjugated antibodies and chemiluminescence (in house reagents). Densitometry measurements were performed using the Chemidoc XRS system with ImageLab software (Bio-rad, Gladesville, NSW, Australia). All images were obtained within the linear range of detection.

Platelet P2RY12 cDNA cloning

Platelets were purified from EDTA-anticoagulated peripheral blood and lysed in Trizol (Life Technologies, Mulgrave, VIC, Australia). Total mRNAs were extracted using the manufacturer's protocol.
The total cDNAs were generated using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) and specific P2RY12 exon 2 PCR performed. The PCR product was purified and cloned into pGEMt vector (Promega, Alexandria, NSW, Australia) for amplification before Sanger sequencing of individual colonies.

Radioligand binding in transfected cell lines

P2Y12 surface receptor expression was determined by ligand binding with [3H]2MeS-ADP (3 Ci mmol–1;1 nM–10 mM), as previously described[6,7].

Construction of P2Y12R constructs

HA/FLAG-tagged P2Y12R constructs were generated as previously described[17]. The R265P and R265W mutations were generated with a Stratagene Quick Change Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) by PCR amplification. DH5a cells were used for transformation, ampicillin-resistant colonies were amplified, and the correct sequence was confirmed by Sanger sequencing.

Cell culture

Human HEK293 cells were transiently transfected (5 μg / 100 mm dish) with N-terminal tagged wild type (WT) P2Y12R or specific variants (R265P and R265W) as previously described6. In co-transfection studies cells were transfected with equivalent levels of HA-tagged (5 μg / 100 mm dish) or FLAG-tagged receptor construct (5 μg / 100 mm dish). Receptor expression was assessed by radioligand binding, western blotting and ELISA , as previously described[18].

Expression and internalization of HA/FLAG-tagged P2Y12R constructs

Surface receptor expression was measured in non-permeabilized cells and total receptor expression was assessed in permeabilized cells (0.1% Triton X100; 30 min) with an anti-HA (HA-11) or anti-FLAG mAb (M2). ADP-dependent tagged surface receptor loss was assessed by ELISA as described previously[18].

P2Y12R function

WT and mutant receptor functions were measured as previously described[17]. Briefly, transfected cells were incubated with 1 µM forsoklin (Sigma-Aldrich, Gillingham, UK) to increase basal cAMP levels in the absence or presence of increasing concentrations of ADP (0.1 nM – 10 µM). cAMP levels were subsequently determined with a cAMP Enzyme Immunoassay Kit, according to the manufacturer's instructions. (Sigma-Aldrich, Gillingham,UK).

Impact of R265P on the receptor conformation

Visualization of the P2Y12R crystal structure (PDB code: 4pxz <PMID: 24784220>) was carried out using PYMOL (Schroedinger, LLC).

Results

A Caucasian family (Fig.1A) of Anglo-Saxon descent consisting of a father (II.2) and his two children (III.3 and III.4) was referred to us for investigation of a possible inherited platelet disorder. They all reported lifelong epistaxis, bruising and abnormal bleeding from minor wounds. The proband (II.2) suffered from severe bleeding following colon polypectomy which required a lot of blood products and tranexamic acid (TA) to be stopped. His son (III.3) experienced also significant bleeding following circumcision requiring heavy compression and TA. The young daughter (III.4) has not reached puberty and has not had surgery. A diagnosis of autosomal dominant bleeding disorder was made. Full blood counts and stained blood films were unremarkable for the proband (II.2) and his children (III.3 and III.4). All three had normal platelet count, normal coagulation screening tests and normal plasma levels of fibrinogen factor VIII and von Willebrand factor (vWF) excluding a coagulation defect or vWF disease. The

calculated bleeding score using the ISTH/SSC bleeding assessment tool {Rodeghiero, 2010 #141} was 9 for II.2, 6 for III.3 and 4 for III.4 .

Identification of a novel P2RY12 G794C mutation

NGS revealed a non-synonymous single G794C substitution on the P2RY12 gene leading to an R265P substitution that was present in father and children and confirmed by Sanger sequencing (Fig.1B)(BankIt2020574-NM_022788.3-MF182351). The PredictSNP server using 6 tools for computational annotation gave a damaging prediction score of 87%.

Subsequently, more family members were tested and the proband's father (I.2) was identified as the carrier (Fig.1A). Interestingly, he recently underwent open heart surgery without any serious bleeding but received high dose TA pre-operatively. Due to his old age and decline in his cognitive functions, a bleeding score could not be calculated.

Platelet function studies

LTA performed on all 4 carriers (I.2, II.2, III.3 and III.4) revealed a severe deficit in ADP-stimulated platelet aggregation (primary wave only) and reduced aggregation to collagen, epinephrine and TRAP, with disaggregation at lower concentrations (Figures 1C, 1D and Table 1). Following ADP activation, platelet α-granule release was reduced as measured by P-selectin exposure and platelet/monocyte aggregate formation; binding of the fibrinogen mimetic antibody Pac-1 was also reduced compared to control representing the lowest percentiles of the normal range (Fig.1F).

P2Y12R R265P expression

Platelet surface expression of the P2Y12 receptor was similar in proband (II.2) and control as measured by flow cytometry (Fig.2A). Similarly, total platelet content was also normal following western-blotting

experiments (Fig.2B). The specificity of P2Y12 receptor antibody used in these studies was validated by using HEK293 cells transfected with FLAG-tagged-P2Y12R in both our flow cytometry and western blotting studies (see Figure 2).

R265P P2Y12R impairs signaling in a dominant negative manner

Further detailed study was undertaken in cells expressing tagged-wild type (WT) and variant receptor constructs. Initial studies assessed if the R265P-P2Y12R variant and WT receptor expressed at comparable levels at the cell surface. Equivalent concentrations of HA-tagged variant or FLAG-tagged WT receptor cDNA were transiently transfected into HEK293 cells. Total expression of the R265P-P2Y12R variant as assessed by ligand binding was similar to WT and that of the related P2Y12R variant R265W (Fig.3A). Further analysis by ELISA in was also undertaken to assess FLAG-tagged WT or HA-tagged R265P variant expression. Total receptor, as assessed in permeabilized HEK293 cells, was comparable between WT and R265P variant P2Y12R (Fig3B). Surface receptor,as assessed in non-permeabilized cells was also equivalent between WT and variant P2Y12R (Fig.3B). Western blotting of cell lysates also revealed comparable total expression of FLAG-WT and HA-R265P variant (Fig.3C). Given the comparable levels of total and surface receptor expression agonist-stimulated receptor function was assessed. Notably, ADP-stimulated inhibition of forskolin-stimulated AC activity (Fig.3D and E) was significantly attenuated for the R265P variant versus WT P2Y12R. The activity of the related P2Y12R variant R265W was also reduced although not to the extent to that of R265P (Fig.3D). ADP promotes agonist-stimulated P2Y12R internalization [19] and is therefore another functional readout of receptor activity. Again, ADP-stimulated surface receptor loss was significantly attenuated in R265P variant versus WT receptor.

In the current study and in previous work from ourselves [6,20] and others [5] there is a significant impairment of receptor function even in patients who heterologously express mutant P2Y12 receptor

variants. The loss of function in these patients is often surprising since they should still express a WT copy of receptor that one would predict could still support normal receptor function. We therefore examined in this study if R265P variant had the potential to act as dominant negative mutant reducing WT receptor activity when they were co-expressed. Initial ELISA (Fig. 3B) and western blotting (Fig. 3C) studies confirmed that when co-transfected with equivalent levels of cDNA there were comparable levels of variant and WT receptor expression. Therefore co-expression of the R265P variant had no apparent effect on either surface or total WT P2Y12 receptor expression. . Intriguingly on a functional level (agonist-dependent signaling (Fig.3E) or surface loss (Fig.3F)) the deficit in R265P variant function was not rescued by WT co-transfection suggesting a dominant negative phenotype. Indeed ADP-stimulated receptor function (Fig.3D and E) was equivalent to R265P P2Y12R variant alone.

P2RY12 gene expression in affected individuals

P2RY12 cDNAs from patients I.2 and II.2 were cloned and sequenced. Surprisingly, only 4/21 clones for I.2 and 6/18 for II.2 were found to be WT (average 25%) while the majority was mutant (75%)(Fig.4A). Dimerization plays an important role in P2Y12R function; from the cDNA data, we extrapolated the amount of the different homodimers and WT dimers only represented less than 10% of total (Fig.4B).

P2Y12R 3D modelling

Protein modelling suggests that the amide proton of R265 makes a hydrogen bond with the carbonyl oxygen of residue 261 (as is normal in a helix). Q263 sidechain makes direct contact with the terminal phosphate group of the ligand (Fig.4C). In an R265P mutant, the hydrogen bond to amino acid 261 will be unable to form because the proline doesn't have an amide proton. As a consequence, the mutation could destabilize this end of helix H6 and thereby not only affect the ligand binding pocket by affecting the positioning of E263 but also prevent the normal docking of the ligand between the transmembrane

domains and consequently impacting on the normal receptor signaling. In contrast, mutation to tryptophan (R265W) does not cause this same degree of disruption withthe 261–265 hydrogen bond still able to form. The nature of the sidechain on the tryptophan is significantly different to the native arginine, however, resulting in a milder phenotype.

Discussion

IPDs due to P2RY12 variants are characterized by lifelong history of excessive bleeding, prolonged bleeding time and severely impaired platelet aggregation responses to ADP. Since the first P2RY12 variant description, the assumption was that heterozygous carriers were asymptomatic until reports of 3 dominant variants[5,6,20]; however the mechanism behind the different genotype/phenotype correlation has not been elucidated. In a Caucasian family, we identified a new dominant negative *P2RY12* variant, responsible for severe bleeding requiring transfusion following minor surgical procedures. We confirmed the role of P2Y12R EL3 in receptor signaling and the importance of amino acid in position 265. Using a cell based model, we have reproduced the dominant negative phenotype and we propose a mechanism linking the genotype to the phenotype.

Despite being rare, the identification of P2RY12 variants has helped identify functionally important regions of this GPCR. Loss of function variants in the EL2-transmembrane domain (TM) 5 region are associated with abnormal ligand binding[6,21], while variants in EL3-TM6, despite normal ligand binding, are associated with abnormal receptor function suggestive of these regions' role in signal transduction[22]. Intracellular regions including EL2 and the extreme C-terminus are associated with changes in receptor activity[17] and traffic[7] (Fig.4D). In 2003, Cattaneo and colleagues reported a patient with lifelong history of easy bruising and bleeding who was a compound heterozygous

R256Q/R265W with normal platelet P2Y12R expression but impaired function. Two asymptomatic offspring of the proband inherited the R265W variant alone and their platelets only had reduced aggregation to low dose ADP[2]. Hoffman et al showed that when substituted, P2Y1R R287, which is equivalent to P2Y12R R265, induced >1000-fold shift in EC50 and suggested that R287 may be involved in an ionic bridge between EL2 and EL3, crucial for receptor activation[23]. Further data supporting the importance of the R265 in the P2Y12R were recently reported in yeast by Ignatovica and colleagues showing the importance of amino acids in positions E181, R256 and R265 in maintaining the function of P2Y12R[24], although, the observed EC50 changes were more modest than in our current study. Zhang and colleagues have shown that R265-P2Y12R variants impairing activation impact on the conformational states of the receptor, rather than on ligand binding[25].

The family described in this paper reported lifelong easy bruising and the father and his son (II.2 and III.3) suffered serious bleeding following surgical procedures only stopped after blood transfusion and TA for the father and a combination of compression and TA for his young son. Platelet aggregation to ADP was markedly impaired in all carriers. Both surface and total expression of P2Y12R was comparable with that from platelets taken from healthy controls. Heterologous expression of FLAG-tagged-WT and HA-R265P in HEK293 cells confirmed our platelet studies with comparable surface and total expression. Importantly although ligand binding to the R265P variant was found not to be effected receptor signalling as assessed by inhibition of forskolin-stimulated AC activity was impaired. . Our data also support earlier studies showing the lower impact of the R265W mutation[3] compared to R265P on receptor signaling. Following binding, ligand docking between the TM domains is critical for receptor-mediated signaling with a stable salt bridge between E188 and R256 linking TM5 and TM6 stabilized by a hydrogen-bond between Q195 and T260[26]. The X-ray crystal structure of P2Y12R bound to 2MeS-ADP15 shows H-bonds between R265 and L261, and between Q263 and the ligand (Fig.4C). The R265P

substitution would disrupt the former H-bond and destabilize the C-terminus of helix-6, thereby likely affecting the ligand binding pocket by repositioning Q263 and in turn impairing receptor signaling.

Dimerization of GPCRs plays an important role in their function[9-12], as demonstrated with clopidogrel, a platelet P2Y12R antagonist and antithrombotic agent, interfering with homo-oligomeric assembly of P2Y12R and inhibiting localization of these complexes into lipid rafts required for receptor activation[27]. An important finding of our study is the disruption of WT P2Y12R function by co-expression with the R265P variant when the receptors were expressed atequivalent levels. Future detailed studies with increasing levels of WT receptor are planned to assess at which point excess WT receptor can effectively overcome the dominant negative effect of the R265P variant and the potential role receptor oligomerization may play in this process.

Platelet mRNA studies have also shown overexpression of the mutant allele in I.2 and II.2 resulting in marked decrease of WT homodimers and suggests differential allelic expression[28]; however, we cannot exclude preferential expression or increased mRNA survival as a driver for the increase in mutant mRNA[29]. Imprinted genes are normally monoallelically expressed so in our family; the 3 fold increase in mutant mRNA compared to WT would favor differential allelic expression rather than imprinting. Our mRNA study showed significantly increased expression of the same mutant allele in the 2 heterozygous individuals tested and that is probably the same in both children. However, differential allelic expression is often associated with variation in transmission of some diseases[28] which has not been observed in this family and would suggest increased mutant mRNA survival further enhancing the dominant phenotype.

In conclusion, recent improvements in high throughput sequencing technologies led to the identification of a novel dominant R265P *P2RY12* variant in a family with absence of irreversible ADP-induced platelet aggregation and severe post-surgical bleeding. As shown by 3D modelling, R265 helps maintaining the correct EL3 structure important for ligand docking and receptor signaling. Our report further highlights

the important role of receptor homodimerization in ADP-P2Y12R signaling and helps explain the dominant negative phenotype observed in patients[5,6,20].

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Conflict-of-interest disclosure: Nothing to disclose

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Authorship

D.R., M-C.M-K. and S.G. carried out mutational screening and analyzed data; S.J.M. M-C.M-K and Q.C. processed biological samples, performed LTA, flow cytometry and analyzed data; S.J.M., J.L.H. and RA generated vectors, conducted the mammalian cell experiments and quantified P2Y12R expression in patient samples; T.K. enrolled patients, provided biological samples and clinical information; J.M. performed the 3D modelling and interpreted the changes; M-C.M-K., S.J.M. D.R. W.S. and C.M.W. designed research, interpreted data, and wrote the manuscript.

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Table 1 Platelet aggregation by light transmission aggregometry over a 8 minute period showing impaired aggregation to ADP (primary wave only) and reduced aggregation to collagen, arachidonic acid, epinephrine and TRAP in affected individual II.2 compared to his unaffected brother II.1 (when full and stable aggregation was observed, higher concentrations of agonists were not tested) (MA: maximum amplitude).

* Disaggregation was observed after reaching the maximum amplitude (MA).
** This individual has been taking aspirin long term.
#: with secondary wave

	II.2 (affected)		I.1 (affected)		II.1 (unaffected)		Controls N=15
Agonists	MA %	Velocity	MA %	Velocity	MA %	Velocity	Range % MA
ADP 2.5 μM	1	9	31*	72	16	59	20-90
ADP 5.0 μM	0	20	29*	63	63	59	59-95 #
ADP 10.0 μM	13*	48	54*	86	/	/	
ADP 20.0 μM	22*	59	57*	90	/	/	
ADP 50.0 μM	21*	61			/	/	
Collagen 0.5 μg/ml	0	0	6	4	87	66	55-90
Collagen 1.0 μg/ml	6*	17			/	/	60-100
Collagen 2.0 μg/ml	52*	46	14	7	/	/	
Collagen 5.0 μg/ml	68	75	49	28	/	/	
AA 0.33 mM	0	4			106	75	10-110
AA 1.6 mM	90	68	17**	11	/	/	75-105
Epinephrin 5.5 μM	23	11	66	50	83	55	10-100
Epinephrin 110 μM	81	42	57	48	/	/	75-105
Risto 0.5 mg/ml	2	7	0	0	0	0	0-4
Risto 1.5 mg/ml	90	77	87	138	75	107	75-100
TRAP 10 μM	14*	57	21*	53	85	97	80-100
TRAP 20 μM	75*	121	74*	116	/	/	80-100

Fig. 1. Phenotype and genotype of a Caucasian family with autosomal dominant IPD.

(A) Family tree showing the transmission of the *P2RY12* variant (Black) and including the year of birth for all individuals tested (individuals not tested are represented in light grey) (B) Sanger sequencing trace demonstrating the G794C variant in *P2RY12* exon 2. (C,D). Platelet light transmission aggregometry for the proband (II.2) showed profound impairment of the ADP-induced aggregation with primary wave only (blue: 2.5 μM, black: 5μM, red 10μM and green: 20μM ADP)(C), and reduced aggregation to collagen and epinephrine (blue: ADP 50 μM, black: collagen 1 μg/ml, red arachidonic acid 1.65μM and green: epinephrine 5.5 μM (D). (E) The level of platelet activation in response to 5μM ADP was measured by flow cytometry and showed reduced P-selectin expression, Pac-1 binding and platelet/monocyte aggregate formation compared to a control (F) representing the lower percentile of the normal range .

Fig. 2. P2Y12R platelet surface expression and total content

(A) Flow cytometry analysis showing surface P2Y12 R levels on platelets from healthy donor (control, left) and patient (right). Surface P2Y12R levels were quantified in terms of geometric mean fluorescence intensity (MFI) from a gated population of 10,000 platelets after accounting for non-specific binding in negative controls. Data displayed show mean of three replicates. We validated the efficacy of the P2Y12 antibody by flow cytometry in vector control versus FLAG-tagged P2Y12R-transfected HEK293 cells. Binding / signal was approximately 3 times higher in transfected versus non-transfected cells (Mean Fluorescent Intensity (- background control) 7520 ± 532 and 2563 ± 322 in FLAG-tagged P2Y12 transfected cells versus vector transfected controls).

(B) Representative immunoblot showing total P2Y12R levels in platelets from healthy donor (control, lane 3) and patient (lane 4). Efficacy of P2Y12R antibody used in these studies was confirmed by using HEK293 cells transiently transfected with FLAG-P2Y12R (lane 2) or vector control (lane 1). Receptor

specific bands (arrows) found in P2Y12R transfected cells and human platelets are highlighted with arrows. Immunoblot of α-tubulin were used as loading control.

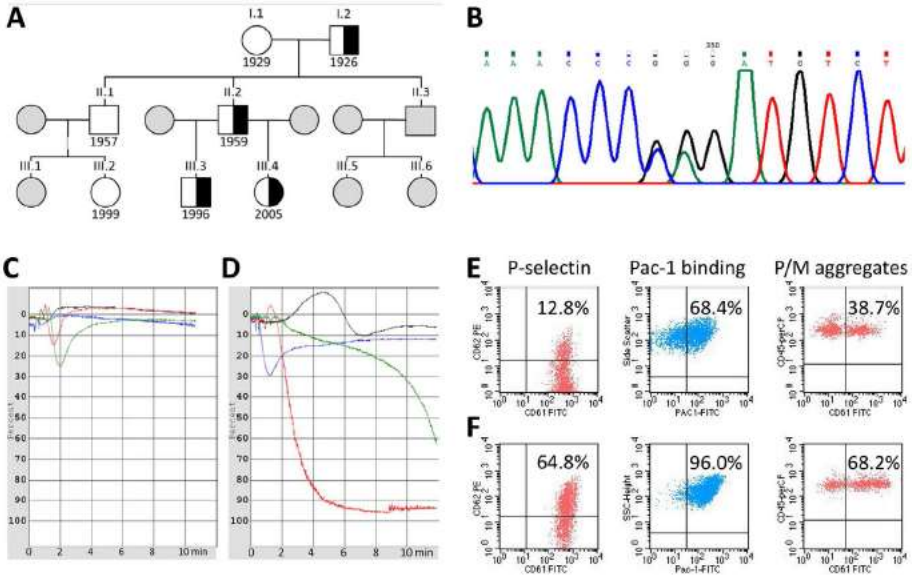
Fig. 3. The R265P P2Y12R variant has impaired receptor function and is able to attenuate wild type P2Y12R function in a dominant negative manner. In these studies HEK293 cells were transiently transfected with equivalent amounts of N-terminal FLAG-tagged wild type (WT), HA-tagged P2Y12R variants (R265P and R265W) or co-transfected with both WT and R265P receptor construct. Receptor levels were measured by either (A) ligand binding using [3H]2MeSADP (0.1-10μM) in the presence of the P2Y12R antagonist AR-C69931MX (1μM) to determine total receptor specific binding in cell homogenates, or ((B) an ELISA based assay or (C) Western Blotting. In (B) FLAG-WT (bars 1-4) expression was assessed by using a FLAG specific antibody. HA-R265P variant (bars 5-8) expression was assessed using an anti-HA specific antibody. Total receptor expression was assessed in permeabilized cells (0.1% triton). Surface receptor expression was assessed in non-permeabilized cells. . Note there was comparable expression of WT and variant receptor construct with no significant change in either total or surface expression of FLAG-WT construct following co-transfection with HA-R265P. In (C) HEK293 cells were transiently transfected with either vector control, FLAG-WT alone, HA-R265P alone or combined FLAG-WT and HA-R265P. Subsequently cell lysates were probed with either a monoclonal anti-FLAG or a polyclonal anti-HA antibody. Receptor specific bands found in receptor transfected cells are highlighted with arrows. Note there was comparable total expression of WT and variant receptor construct with no significant change in FLAG-WT expression following co-transfection with HA-R265P. Immunoblot of α-tubulin were used as loading control.

(D, E and F) Agonist-stimulated R265P variant function is significantly impaired versus WT P2Y12R. (D and E) Agonist (ADP; 0.1nM to 1μM)–dependent inhibition of forskolin (1μM; 10 minutes)–stimulated

adenylyl cyclase activity was assessed in HEK293 cells transiently expressing WT and mutant variant receptor constructs. (F) ADP (10 μM)-stimulated R265P variant surface receptor loss as assessed by ELISA in non-permeabilized cells was attenuated versus WT P2Y12R. Note in (E) and (F) Co-expression of WT with R265P variant significantly attenuated WT agonist-stimulated receptor function with both signalling (E) and surface receptor loss (F) effectively reduced to R265P variant levels. Data represent means (±SEM) of at least 4 independent experiments.

Fig. 4. (A) Platelet *P2RY12* cDNAs from 2 affected individuals (I.2 and II.2) were cloned and sequenced. The number of WT and mutant (M) clones is expressed as percentage of total and (B) the P2Y12R dimer content has been extrapolated (B), correlating the genotype to the phenotype. (C) The crystal structure of P2Y12R (grey ribbon) bound to 2MeS-ADP (orange) is shown, highlighting the hydrogen bonds between R265 and L261 (blue label), and between Q263 and the 2Me-SADP (red label). The R265P mutation could destabilise the C-terminal end of helix H6 and thereby affect the ligand binding pocket by affecting the positioning of Q263. D: This snake plot shows the extracellular loop (EL) and transmembrane (TM) domains as well as the recessive (blue circles) and dominant (red circles) P2Y12R pathogenic point mutations identified in patients (Adapted from [22]). Three out of the four dominant mutations involve a change from or to a proline.

Figure 1



Phenotype and genotype of a Caucasian family with autosomal dominant IPD.
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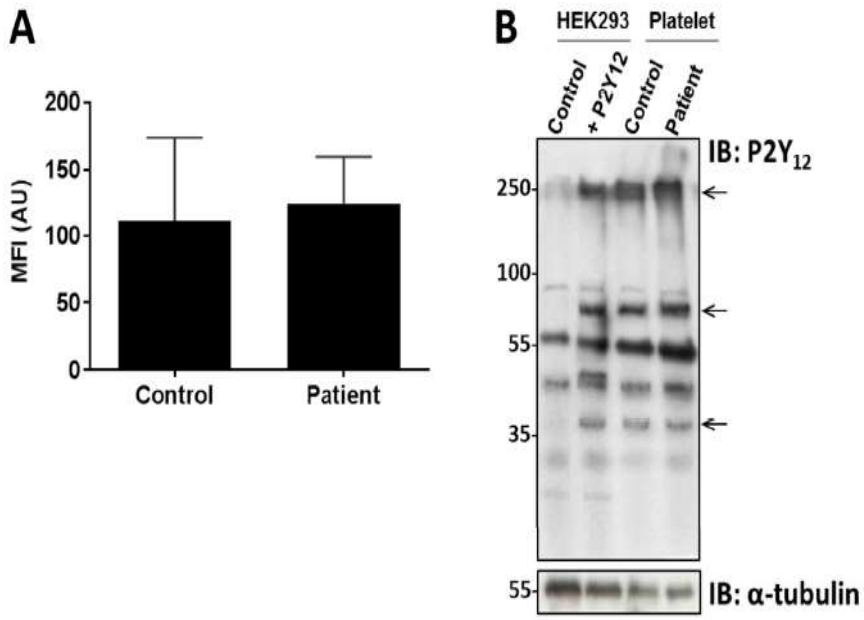
Figure 2



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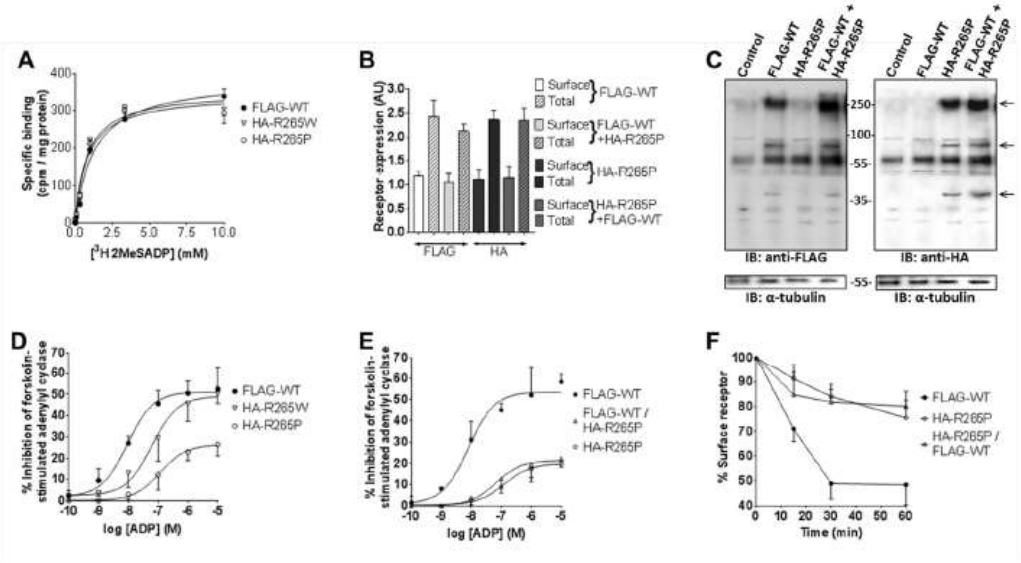

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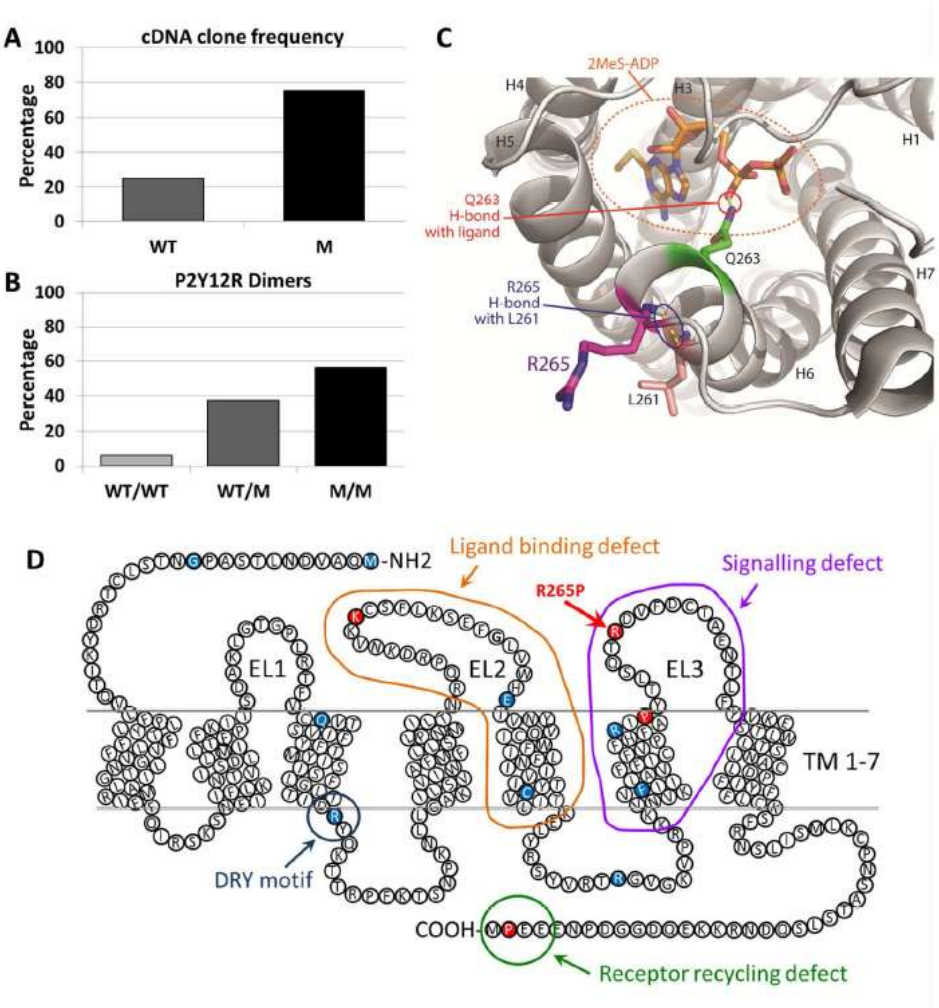

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